Synthesis[1] of Ring-Labeled L-Thyroxine-C^{14}

MELVIN J. GORTATOWSKI, LINDY F. KUMAGAI, AND CHARLES D. WEST

Departments of Biological Chemistry and Medicine, University of Utah College of Medicine and the Veterans Administration Hospital, Salt Lake City, Utah

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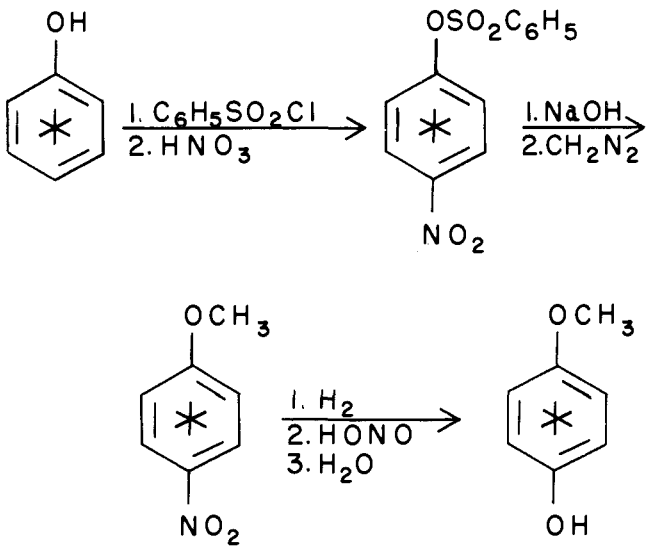
Studies with I^{131}-labeled thyroxine and carboxyl C^{14}-labeled thyroxine have afforded valuable information on the metabolism of this important thyroid hormone. Because the I^{131}- and the C^{14}-carboxyl labels are readily removed physiologically, our knowledge about the metabolic fate of the diphenyl ether moiety of the molecule is incomplete. To investigate this aspect of thyroxine metabolism, L-thyroxine labeled with C^{14} in the phenolic ring has been synthesized.

In order to utilize a previously reported[2] method for the convenient preparation of L-thyroxine from *p*-methoxyphenol, it was necessary to prepare this intermediate from phenol-C^{14} by the reaction scheme shown in Chart I. Then, by modification of the Chalmers, *et al.*,[2] method for the condensation of N-acetyl-3,5-dinitro-L-tyrosine ethyl ester with *p*-methoxyphenol and conversion of the condensation product to L-thyroxine, it was possible to prepare L-thyroxine-C^{14} in twelve steps in an over-all yield of 2.6%. The details are reported in this communication.

CHART I



Experimental[3]

Phenyl-C^{14} Benzenesulfonate.—To a stirred solution of phenol-C^{14} (uniformly labeled, specific activity, 1.3 mc./mmole,[4] 216 mg., 2.3 mmoles) in anhydrous pyridine (1 ml.) was added a solution of redistilled benzenesulfonyl chloride (460 mg., 2.6 mmoles) in anhydrous pyridine (1.1 ml.) over a period of 5 min. while the temperature was maintained at 25–30°. Stirring was continued at 25–30° for 1 hr., then the mixture was allowed to stand overnight at room temperature (25°). Ice (4 g.) was added to the reaction mixture. After adjustment to pH 1 with concentrated hydrochloric acid, the turbid solution was saturated with sodium chloride, and the product was extracted with ten 3-ml. portions of benzene. Evaporation of the benzene afforded crude phenyl-C^{14} benzenesulfonate (560 mg., 104%) as a colorless oil (lit.[5] m.p. 34–35°).

***p*-Nitrophenyl-C^{14} Benzenesulfonate.**—The crude phenyl-C^{14} benzenesulfonate (assume 2.3 mmoles) was cooled below 0°, and concentrated sulfuric acid (0.47 ml.) was added slowly (5 min.) with manual stirring. To the mixture was added finely pulverized potassium nitrate (293 mg., 2.9 mmoles) with manual stirring over a period of 30 min. while the temperature was maintained at 0° or below. The nearly dry cream-colored reaction mixture was then warmed to 40–50° (oil bath) for an additional 30 min. with periodic stirring, cooled, and allowed to stand at room temperature (25°) for 1 hr. The mixture was cooled below 0°, and ice (4 g.) was added with stirring. The aqueous phase was saturated with sodium chloride and the product extracted with eight 4-ml. portions of ether. Evaporation of the ether afforded a residue which was rendered anhydrous by three evaporations of its solutions in anhydrous benzene (10 ml. each). The oily yellow solid (629 mg., m.p. 47–66°) was recrystallized from absolute methanol (1.4 ml.). The product was collected, washed with two 0.25-ml. portions of methanol, and dried *in vacuo* over phosphorus pentoxide. The light yellow prisms of *p*-nitrophenyl-C^{14} benzenesulfonate (404 mg., 63% based on phenol-C^{14}) melted at 82–83°, lit.[6] m.p. 82°), specific activity, 1.38 mc./mmole.

***p*-Nitrophenol-C^{14}.**—A solution of *p*-nitrophenyl-C^{14} benzenesulfonate (404 mg., 1.45 mmoles) in 2 *N* sodium hydroxide (5 ml.) and ethanol (15 ml.) was refluxed for 1 hr. The ethanol was evaporated, and the resulting aqueous solution was acidified to pH 1 with concentrated hydrochloric acid, then saturated with sodium chloride, and extracted with ten 10-ml. portions of ether. The ether was evaporated and the residue rendered anhydrous by two evaporations of its solutions in anhydrous benzene (15 ml. each). The resulting cream-colored needles of *p*-nitrophenol-C^{14} (202 mg., 100%) melted at 109–110.5°, lit.[5] m.p. 114°.

***p*-Nitroanisole-C^{14}.**—*p*-Nitrophenol-C^{14} (202 mg., 1.45 mmoles) was dissolved in anhydrous ether (10 ml.) containing a trace (about 1 mg.) of freshly prepared aluminum *n*-butoxide.[8] To the mixture was added a dry solution of diazomethane in ether[9] at a fairly rapid rate at room temperature. The reaction vessel was loosely stoppered and allowed to stand at room temperature (24°) for 1 hr. The excess diazomethane was destroyed by addition of glacial acetic acid (1 ml.) with stirring, and the pale yellow ether solution was extracted successively with four 10-ml. portions of *N* sodium hydroxide and three 10-ml. portions of saturated sodium chloride. The combined sodium hydroxide and sodium chloride extracts were reserved for recovery of unchanged *p*-nitrophenol-C^{14}. The ether phase was evaporated[10] (12 mm., 45°), and the residual oily solid was rendered anhydrous by two evaporations of its solutions in heptane (15 ml. each). The cream-colored feathery needles of *p*-nitroanisole-C^{14} (128 mg.) melted at 46–49°, lit.[11] m.p. 54°.

The unchanged *p*-nitrophenol-C^{14} was recovered from the combined sodium chloride and sodium hydroxide extracts by acidification to pH 1 with concentrated hydrochloric acid, saturation with sodium chloride, and extraction with ten 10-ml. portions of ether. The ether was evaporated and the residue rendered anhydrous by two evaporations of its solutions in anhydrous benzene (10 ml. each).

The recovered *p*-nitrophenol-C^{14} (89 mg., m.p. 109–110.5°) was methylated in a manner similar to that used above with the same amount of diazomethane overnight at room temperature. The total yield of *p*-nitroanisole-C^{14} obtained after three methylations was 223 mg. (100%).

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(1) This work has been supported in part by a grant (M-2530) from the National Institutes of Health, U. S. Public Health Service.

(2) J. R. Chalmers, G. T. Dickson, J. Elks, and B. A. Heus, *J. Chem. Soc.*, 3424 (1949).

(3) Melting points were determined on a Fisher–Johns hot stage apparatus and are corrected.

(4) Procured from New England Nuclear Corporation, Boston, Massachusetts.

(5) R. Otto, *Ber.*, **19**, 1833 (1886).

(6) C. Schiaparelli, *Gazz. Chim. Ital.*, **11**, 77 (1881).

(7) R. Schiff, *Ann.*, **223**, 263 (1884).

(8) H. Meerwein and G. Hinz, *Ann.*, **484**, 1 (1930).

(9) Diazomethane was prepared from N-nitrosomethylurea (0.6 g.), 40% potassium hydroxide (2.4 ml.), and anhydrous ether (30 ml.).

(10) Because of its high volatility, the product was not kept under vacuum longer than was necessary to remove the solvent.

(11) I. Heilbron and H. M. Bunbury, "Dictionary of Organic Compounds," Vol. III, Oxford University Press, New York, N. Y., 1953, p. 632.

p-Aminoanisole-C^{14}.—A solution of *p*-nitroanisole-C^{14} (223 mg., 1.45 mmoles) in 95% ethanol (15 ml.) containing platinum oxide catalyst (20 mg.) was hydrogenated at slightly above atmospheric pressure at 25°. After the reduction was complete (1 hr.), the solution was filtered under an atmosphere of CO_2 into a flask containing 6.0 *N* sulfuric acid (1.0 ml.). The catalyst and reaction flask were rinsed with ethanol and the rinsings filtered and transferred to the main solution. Evaporation of the ethanol afforded crystalline *p*-aminoanisole-C^{14} hydrogen sulfate. The product was freed from last traces of ethanol by three evaporations of its suspensions in anhydrous benzene (10 ml. each).

p-Methoxyphenol-C^{14}.—The light tan *p*-aminoanisole-C^{14} hydrogen sulfate (1.45 mmoles, assume 100% reduction) was suspended in 3 *M* sulfuric acid (2.5 ml.) and diazotized at 0 to 5° with sodium nitrite (102 mg., 1.48 mmoles) in water (1.2 ml.). The diazonium solution was stirred at 0 to 5° for 15 min., then the excess nitrite was destroyed with urea. The diazonium salt was hydrolyzed by boiling it with 6 *N* sulfuric acid (50 ml.) for 30 min. The reaction mixture was cooled and extracted with four 20-ml. portions of peroxide-free ether. After the extraction, the dissolved ether was expelled from the aqueous phase by careful heating and the aqueous mixture refluxed again for 30 min. This process of boiling for 30 min., ether extraction, expulsion of dissolved ether from reaction mixture, and refluxing the hydrolysate again was carried out a total of eight times.

The combined yellow ether extract was concentrated to about 30 ml., and the resulting ether solution was washed with three 5-ml. portions of saturated sodium chloride solution. The product was then extracted with four 10-ml. portions of *N* sodium hydroxide. The sodium hydroxide extracts were combined and acidified to pH 1 with concentrated hydrochloric acid, saturated with sodium chloride, and extracted with ten 10-ml. portions of ether. The ether was evaporated and the residual oil rendered anhydrous by two evaporations of its solutions in dry benzene (10 ml. each). The crude red-brown semisolid *p*-methoxyphenol-C^{14} amounted to 177 mg. (98% based on *p*-nitroanisole-C^{14} or 62%[12] based on phenol-C^{14}). A melting point determination was not practical, lit.[13] m.p. 53°. A paper strip (Whatman No. 1) chromatogram was developed by ascending technique in benzene–propionic acid–water (100:75:5). A single radioactive peak was obtained which corresponded to authentic nonradioactive *p*-methoxyphenol at R_f 0.71. On spraying the chromatogram with diazotized sulfanilic acid, a single spot developed which corresponded with the radioactive peak.

N-Acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-dinitrophenyl]-L-alanine Ethyl Ester.—The procedure of Chalmers, *et al.*,[2] was followed with modification of the amounts of reactants. From N-acetyl-3,5-dinitro-L-tyrosine ethyl ester[2] (470 mg., 1.38 mmoles), *p*-toluenesulfonyl chloride (263 mg., 1.38 mmoles), anhydrous pyridine (1 ml.), and *p*-methoxyphenol-C^{14} (177 mg., 1.42 mmoles) dissolved in pyridine (1.6 ml.) there was obtained the yellow-brown crude N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-dinitrophenyl]-L-alanine ethyl ester (405 mg., 66% based on tyrosine derivative) which melted at 93–100° (softened at 68°).

The unchanged *p*-methoxyphenol-C^{14} was recovered from the sodium hydroxide extracts by acidification to pH 1 with concentrated hydrochloric acid, saturation with sodium chloride, and extraction with ten 10-ml. portions of ether. The combined ether extracts were extracted with three 10-ml. portions of *N* sodium bicarbonate. The ether solution was filtered through a cotton plug and the ether evaporated. The residual oil was rendered anhydrous by two evaporations of its solutions in anhydrous benzene (10 ml. each). The recovered *p*-methoxyphenol-C^{14} (33.5 mg.) remained as a yellow oil. By carrying out a similar condensation on the recovered *p*-methoxyphenol-C^{14}, there was obtained a second crop of the dinitrodiphenyl ether derivative (85 mg.), m.p. 89–98° (softened at 83°). The two crops of crude condensation product were combined (490 mg.), recrystallized from *t*-butyl alcohol (1.5 ml.), and dried over phosphorus pentoxide *in vacuo*.

The recrystallized N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-dinitrophenyl]-L-alanine ethyl ester (280 mg., m.p. 88–98°) was dissolved in anhydrous benzene (3 ml.) and chromatographed on a column of Woelm alumina (neutral, grade 1) measuring 100 × 31 mm. The elution was carried out with a mixture of benzene and chloroform as shown in Table I. Final stripping was accomplished with ethanol and methanol.

TABLE I

ELUENT COMPOSITION FOR ALUMINA COLUMN CHROMATOGRAPHY OF DINITRODIPHENYL ETHER DERIVATIVE

Fraction	Volume, ml.	Benzene, ml.	Chloroform, ml.	Radioactivity[a]
IA	50	50	0	Trace
IIA	100	90	10	Trace
IIIA	100	80	20	Trace
IVAa	350	175	175	Trace
IVAb	100	50	50	High
VA	50	25	25	High
VIA	300	0	300	High
VIIA	200	Absolute ethanol		High
VIIIA	200	Absolute methanol		High

[a] Aliquots were counted in the Packard Tri-Carb liquid scintillation counter.

Fraction IVAb contained the bright yellow band from the column characteristic of the dinitrodiphenyl ether derivative. Fractions VA, VIA, VIIA, and VIIIA each afforded on evaporation small amounts of uncrystallizable oils and gums ranging in color from yellow to brown. These radioactive by-products from the condensation reaction were not investigated.

Evaporation of solvent from fraction IVAb afforded the dinitrodiphenyl ether derivative (215 mg.) which was recrystallized from *t*-butyl alcohol (1 ml.), washed with two 0.1-ml. portions of *t*-butyl alcohol, and dried *in vacuo* over phosphorus pentoxide. The light yellow N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-dinitrophenyl]-L-alanine ethyl ester (158 mg., 15% based on phenol-C^{14}) melted at 98–101° (softens 95°), lit.[2] m.p. 109–110°.

N-Acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-diaminophenyl]-L-alanine Ethyl Ester.—A solution of N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-dinitrophenyl]-L-alanine ethyl ester (112 mg., 0.25 mmole) in 95% ethanol (10 ml.) containing platinum oxide catalyst (10 mg.) was hydrogenated at slightly above atmospheric pressure at room temperature (25°). During the reduction, the system was protected from excessive light. After the reduction was complete (about 2 hr.), the mixture was cooled in ice, the flask was evacuated, and glacial acetic acid (0.3 ml.) was added with stirring. The flask was flushed with CO_2, and all subsequent manipulations were conducted in an atmosphere of CO_2. The reaction mixture was filtered, and the solvent was evaporated. The residual pale yellow oil was rendered anhydrous by evaporating its solution in absolute ethanol (25 ml.) and finally rendered alcohol-free by two evaporations of its solutions in anhydrous benzene (25 ml. each). The resulting yellow viscous oil of N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-diaminophenyl]-L-alanine ethyl ester diacetate was used without purification.

N-Acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-diiodophenyl]-L-alanine Ethyl Ester.—A slightly modified[14] procedure of Chalmers, *et al.*,[2] was followed with proportional reduction in the scale of materials throughout. From the crude N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-diaminophenyl]-L-alanine ethyl ester diacetate (0.25 mmole) obtained from the preceding reduction, there was obtained light buff colored prisms of N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-diiodophenyl]-L-alanine ethyl ester (109 mg., 72% based on the dinitrodiphenyl ether derivative) which melted at 136–137°, lit.[2] m.p. 143–144°.

3,5-Diiodo-L-thyronine-C^{14}.—The procedure of Chalmers, *et al.*,[2] was followed with proportional reduction in the scale of reactants throughout. From N-acetyl-3-[4-(4-methoxyphenoxy-C^{14})-3,5-diiodophenyl]-L-alanine ethyl ester (97 mg., 0.16 mmole), hydriodic acid (freshly distilled 57%, 0.25 ml.), and glacial acetic acid (0.25 ml.), there was obtained crude white 3,5-diodo-L-thyronine-C^{14} (45 mg., 54% based on 3,5-diiodo derivative) which melted at 244° dec., lit.[2] m.p. 255° dec. It was used without purification.

(12) Probably not a valid representation of degree of conversion because of the impure nature of the product.

(13) H. Hlasiwetz and J. Habermann, *Ann.*, **177**, 339 (1875).

(14) After the finely pulverized sodium nitrite was added to concentrated sulfuric acid, the mixture was warmed to 70° until solution was complete. It was then cooled to 0° and cautiously diluted with glacial acetic acid.

L-Thyroxine-C^{14}.—The method of Chalmers, *et al.*,[2] was followed. From 3,5-diiodo-L-thyronine-C^{14} (26 mg., 0.05 mmole), 33% aqueous ethylamine (0.26 ml.), and a 1.9 *N* solution (0.11 ml.) of iodine in concentrated potassium iodide, there was obtained crude L-thyroxine-C^{14}. Precipitation from a mixture of 95% ethanol (1 ml.) and 2 *N* sodium hydroxide (0.5 ml.) afforded pure white crystalline L-thyroxine-C^{14} (17 mg., 44% based on 3,5-diiodo-L-thyronine-C^{14}, 2.6% based on phenol-C^{14}). It melted at 227–228° dec.,[15] specific activity 1.55 μc./mg.

Characterization of L-Thyroxine-C^{14}. A. Paper Chromatography.—The L-thyroxine-C^{14} was compared with nonradioactive and I^{131}-labeled L-thyroxine by descending technique in three different solvent systems: *t*-amyl alcohol saturated with 2 *N* ammonium hydroxide, *n*-butyl alcohol–*p*-dioxane–2 *N* ammonium hydroxide (4:1:5), and *n*-butyl alcohol–acetic acid–water (4:1:5). The R_f values were 0.26, 0.48, and 0.85, respectively. A single radioactive peak corresponding to nonradioactive L-thyroxine was observed in each case.

B. Biological Activity.—The L-thyroxine-C^{14} exhibited biological activity equivalent to authentic L-thyroxine when tested by the inhibition of propylthiouracil-induced goiters in rats[16] and the suppression of thyroidal iodine-131 uptake.[17]

Acknowledgment.—The authors wish to express appreciation for the valuable technical assistance of Eveline Bruenger.

(15) The sample was placed on hot stage preheated to 220° and heated at the rate of 4°/min., lit.[2] m.p. 233–235° dec. The optical rotation as determined on a sample of nonradioactive L-thyroxine prepared in the same way from nonradioactive phenol was $[\alpha]_D^{27} = -5.6°$ (*c*, 2.2 in a 1:2 mixture of *N* sodium hydroxide and ethyl alcohol).

(16) (a) E. W. Dempsey and E. B. Astwood, *Endocrinology*, **32**, 509 (1943); (b) C. A. Plamondon, H. A. Selenkow, J. C. Wiswell, and S. P. Asper, Jr., *Bull. Johns Hopkins Hosp.*, **102**, 88 (1958).

(17) W. C. Money, R. I. Meltzer, D. Feldman, and R. W. Rawson, *Endocrinology*, **64**, 123 (1959).

Bicyclic Imides and Isoindolines[1]

CHARLES H. GROGAN AND LEONARD M. RICE

Georgetown University Medical Center, Washington, D. C.

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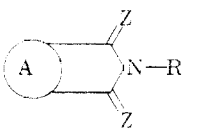
In view of our previous extensive work on isoindoles, isoindolines, the corresponding intermediate imides,[2,3] and other azabicyclic systems,[4] it was desired to screen representative types and derivatives of these systems in the primary rodent tumor and tissue culture screens of the Cancer Chemotherapy National Service Center.[5] Accordingly, a cooperative arrangement was worked out whereby sufficient quantities of these compounds could be made available for the anticancer primary screens.

During the course of this work a number of new derivatives of these ring systems were prepared in addition to those previously reported. Since previous work had shown a high physiological activity, accompanied by low toxicity,[6] in derivatives of the 4,7-epoxyisoindoline ring system, most of the new derivatives reported herein were derived from this nucleus. These derivatives, and some related imides and isoindolines, are listed in Table I together with pertinent physical data.

The imides were prepared by reaction of the appropriate primary amines, with or without solvent depending on the volatility and solubility of the amine, with the desired dicarboxylic acid anhydrides, followed by cyclization of the initially formed amic acid to the corresponding imide.[2] The isoindoline bases were obtained by reduction of the imides with lithium aluminum hydride in absolute ether. In all cases the reduction proceeded smoothly and gave good yields of the desired products except when the N substituent was hydrogen. In this case the yield was considerably reduced (from 80–95% to around 50%).

Representative compounds were submitted to and screened under the auspices of the Cancer Chemotherapy National Service Center in the primary rodent tumor screens (consisting of murine sarcoma 180, adenocarcinoma 755, and lymphoid leukemia 1210). A number of the compounds were also assayed for growth inhibitory activity against the KB cell line in tissue culture.

Isoindolines and other azabicyclic compounds resynthesized for screening are shown by the general formula



wherein the following A ring systems were represented: (1) benzene; (2) cyclohexane; (3) *cis*-Δ^4-cyclohexene; (4) 3,4,5,6-tetrachlorobenzene; (5) 5-methyl-*cis*-Δ^4-cyclohexene; (6) 3,6-epoxycyclohexane; (7) 3,6-methano-Δ^4-cyclohexene; (8) 3-methyl-3,6-epoxycyclohexane; (9) cyclobutane; (10) 1,2,2-trimethylcyclopentane. Z was oxygen (intermediate imides) or 2H (isoindolines or other azabicyclic bases). R was varied to include hydrogen, alkyl groups from 1 to 10 atoms, dialkylaminoalkyl, and heterocyclic alkyl groups. In the latter two side-chain types, R was the composite grouping $-(CH_2)_nNR'R'$ wherein n was varied from 2 to 6; R′ alkyl groups containing from 1 to 6 atoms, or the grouping NR'_2 consisted of the heterocycles morpholine, piperidine, or pyrrolidine. In all cases the isoindolines and other azabicyclic structures were submitted in the form of their acid addition (usually hydrochlorides) salts, to ascertain if activity existed in the base itself, and as quaternary salts, usually as the methonium iodide. Where dialkylaminoalkyl or heterocyclic alkyl side chains existed, presenting a basic nitrogen atom in the side chain, the acid addition and quaternary salts of the imides were also submitted.

An analysis of the screening data for some 100 compounds of the type submitted showed that none had significant activity against leukemia L-1210 (a 25% increase in life span). The hydrogen imides of the various dicarboxylic anhydrides employed were prepared and submitted since tetrahydrophthalimide showed a 24% increase in life span. None of the other imides was as active as tetrahydrophthalimide. None of the alkylimides, dialkylaminoalkyl, or heterocyclic alkyl imides (their acid addition and quaternary salts) passed any of the stages in the assay against

(1) Supported in part by the Cancer Chemotherapy National Service Center, under contract SA-43-ph-2417, and the Geschickter Fund for Medical Research, Inc.

(2) L. M. Rice, E. E. Reid, and C. H. Grogan, *J. Org. Chem.*, **19**, 881 (1954).

(3) L. M. Rice, C. H. Grogan, and E. E. Reid, *J. Am. Chem. Soc.*, **75**, 2011 (1953).

(4) C. H. Grogan and L. M. Rice, *J. Org. Chem.*, **22**, 1223 (1957).

(5) *Cancer Chemotherapy Rept.*, **1**, 13 (1959).

(6) C. H. Grogan and L. M. Rice, U. S. Patent 2,781,199 (1957).